Expression and regulation of endothelin-1 and its receptors in human penile smooth muscle cells

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We report for the first time that penile smooth muscle cells (SMC) not only respond to, but also synthesize, endothelin-1 (ET-1), one of the main regulators of SMC activity. Immunohistochemical studies indicated that, beside endothelial cells (EC), SMC of the human adult and fetal penis also express ET-1 and its converting enzyme, ECE-1. Accordingly, cultures of adult penile stromal cells express these genes. We also prepared and characterized penile SMC from human fetuses. These cells express SMC specific markers such as α smooth muscle actin and phosphodiesterase type 5A3 along with hallmarks of androgen-dependent cells (androgen receptor and 5α reductase type 2). Human fetal penile SMC (hfPSMC) are immunopositive for ET-1 and release ET-1. ET-1 expression in hfPSMC was strongly increased by several factors such as transforming growth factor-β1 (TGF-β1), interleukin-1α (IL-1α), ET-1 itself and prolonged (24 h) hypoxia. This latter condition not only affected ET-1 expression but also responsiveness. While at normal oxygen tension, hfPSMC responded to ET-1 with a decreased proliferation mediated by the endothelin-A receptors and TGF-β1; however, during hypoxia, ET-1 stimulated cell growth. Accordingly, prolonged hypoxia up-regulated endothelin-B receptor mRNA expression. In conclusion, our results indicate that in penile tissues SMC produce ET-1 and that such production is modulated by factors involved in penile physiology and tissue remodelling. In addition, the hfPSMC we have characterized might be a useful model for studying biochemical aspects of the human erectile process *in vitro*.

Key words: corpus cavernosum/endothelin/erection//hypoxia/smooth muscle cells

Introduction

It is widely accepted that nitric oxide (NO) is the principal neurotransmitter causing penile smooth muscle relaxation, whereas the predominant contractile agent is noradrenaline (NA) (Maggi *et al.*, 2000a; Andersson, 2001). During cavernous relaxation there is a dramatic increase in penile blood flow that allows penile erection and a consistent increase in tissue oxygenation. Conversely, when penile vasculature is in the contracted state, the arterial blood inflow is more limited and, therefore, in the flaccid, non-erect state, tissue oxygenation is greatly decreased, reaching venous-like values. Beside NA, during the last 10 years it has also been suggested that endothelin-1 (ET-1) participates in maintaining the penis in a flaccid state (Mills *et al.*, 2001b). ET-1 belongs to a family of potent vasoactive peptides, which are mainly synthesized and released by several types of endothelial cells (EC), including the cavernous ones (Saenz de Tejada *et al.*, 1991). ET-1 is produced from the precursor prepro-ET-1, which is cleaved by a specific endopeptidase into a 38 amino acid big-ET-1. Big-ET-1 is subsequently cleaved into the mature 21 amino acid peptide ET-1 by an ET-converting enzyme, ECE-1 (Rubanyi and Plokoff, 1994). Although ET-1 is considered the most potent and long-lasting vasoconstrictor agent (Davenport, 2002), in many vascular systems, ET-1 may induce both contraction and relaxation. The underlying basis of this double action consists of the presence of two

distinct receptor subtypes: the vasoconstrictor endothelin-A receptor (ETA), which binds ET-1 selectively, and the vasodilator endothelin-B (ETB) subtype, which shows similar affinity for all the ET isopeptides (Davenport, 2002). The presence of ET-1 receptors has been reported in cavernosal tissue of different animal species (Saenz de Tejada *et al.*, 1991; Holmquist *et al.*, 1992; Bell *et al.*, 1995; Sullivan *et al.*, 1997; Khan *et al.*, 1999; Filippi *et al.*, 2002), and injection of ET into the penile vasculature can indeed cause both vasoconstriction and vasodilation in the rat penis (Ari *et al.*, 1996). However, the ETA-mediated cavernous vasoconstriction seems to be predominant *in vivo*, because ETA receptors, but not ETB receptor antagonists, are able to prevent the ET-1-induced rise in intracavernosal pressure in basal conditions or even after sub-maximal ganglionic stimulation (Dai *et al.*, 2000). In the penis, the ETA receptor mediates its biological effect (Dai *et al.*, 2000; Kim *et al.*, 2002) through the activation of either inositol triphosphate/calcium or RhoA/Rho kinase pathways (Mills *et al.*, 2001a), while the ETB receptor mediates vasodilation via release of NO from cavernous EC (Sakurai *et al.*, 1992; Ari *et al.*, 1996; Haynes and Webb, 1998; Schiffrin and Touyz, 1998).

It is generally accepted that EC are the main source of ET-1. However, there is much evidence indicating that ET-1 can also be produced by a wide variety of cell types (Rubanyi and Plokoff, 1994),

including smooth muscle cells (SMC) (Resink *et al*., 1990). Although basal ET-1 secretion from vascular SMC is estimated to represent just 10–30% of the amount secreted by the corresponding EC, its gene and protein expression might be strongly increased by several agents, including TGF-β, thus reaching levels compatible with EC ET-1 production (Sugo *et al*., 2001). Contradictory results have been reported until now on ET-1 production by SMC of the penis. ET-1 mRNA has not been detected by Northern analysis in human penile SMC (Saenz de Tejada *et al*., 1991), but ET-1 protein has been localized in human (Saenz de Tejada *et al*., 1991) and rabbit (Sullivan *et al*., 1997) corpus cavernosum stromal cells.

The aim of the present study was to investigate the expression of ET-1 and its receptors in the human penis, focusing on the possibility that penile smooth muscle cells are not only the target but also an ancillary source of ET-1, as has been demonstrated in other SMC. We therefore studied gene and protein expression of the ET system (ET-1, its specific converting enzyme ECE-1 and both subtypes of ET receptors) in human adult and fetal penile tissue. Experiments with tissue culture were also performed to investigate the principal regulators of the ET system in the penis.

Materials and methods

Chemicals and antibodies

ET-1, ET-3, the ETA-selective antagonist cyclo-[D-Trp- D-Asp-D-Val-Leu] (BQ123), the ETB-selective antagonist *N-cis*-2,6-dimethylpiperidinocarbonyl-γ-MeLeu-D-Trp(MeOCO)-D-Nle-OH Na (BQ788) and the ETB selective agonist Suc-[Glu[9], Ala[11,15]]-ET-1 (8,21) (IRL1620) were obtained from Novabiochem (Switzerland). The polyclonal antibody (Ab) to ET-1 (RAS 6901) was purchased from Peninsula Laboratories (San Carlos, CA, USA). The monoclonal Ab to ET-1 (clone TR.ET.48.5) was purchased from Affinity Bioreagents (Nashanic Station, NJ, USA). Dr Yanagisawa (Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas) provided the Ab against ECE-1. This Ab was produced by immunizing rabbits with a synthetic peptide, CPPGSPMNPHHKCEVW, corresponding to the C-terminal 16 amino acids of bovine ECE-1. Interleukin-1α (IL-1α) and transforming growth factor-β1 (TGF-β1) were obtained from Calbiochem (La Jolla, CA, USA). Polyclonal Ab to TGF-β (pan-specific TGF-β Ab) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Polyclonal Ab to androgen receptor (AR) and the monoclonal Ab to α smooth muscle actin were purchased from Santa Cruz (Santa Cruz, CA, USA). Reagents and medium for cell cultures, reagents for immunocytochemistry, for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), peroxidase-conjugated secondary Ab, *N*-octyl β-D-glucopyranoside (NOG), enhancer 2(*p*-toluidino)naphthalene-6-sulphonic acid (TNS) and NADPH were all obtained from Sigma Chemical Co (St Louis, MO, USA). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). [^{125}I]ET-1, [1,2,6,7-[3]H₄]testosterone, [^{3}H]-R1881, and deoxycytidine 5′-[α-^{32}P]triphosphate were purchased from NEN Life Science (Boston, MA, USA).

Corpus cavernosum preparations

Human corpora cavernosa were obtained from impotent men at the time of penile prosthesis implantation (*n* = 9). After surgery, biopsies of corpus cavernosum were immediately placed in cold Krebs solution and transported to the laboratory for in-vitro experiments. Human strips were vertically mounted under 1.8 g resting tension in organ chambers containing 10 ml Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2 at pH 7.4. The solution had the following composition (mmol/l): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. The preparations were allowed to equilibrate for ⩾90 min; during this period the bath medium was replaced every 15 min. Changes in isometric tension were recorded on a chart polygraph. Drug cumulative concentrations were added, at 7 min intervals, to the bath in order to obtain a concentration-dependent contractile curve; a 15–30 min pretreatment with selected antagonists was performed before repeating the concentration–response curve for ET-1. The increase recorded

in the presence of different concentrations of the agonist was expressed as percentage of maximal KCl (80 mmol/l)-induced response, taken as 100%. The high potassium salt solution was made by equimolar substitution of sodium by potassium.

Immunohistochemistry

Immunohistochemical studies were carried out as previously described (Maggi *et al*., 1991). For studies in tissues, adult and fetal penile sections (fixed in Bouin's solution and embedded in paraffin) were incubated with polyclonal or monoclonal ET-1 antisera (diluted 1:1500 and 1:100 respectively). The sections were then incubated with the IgG peroxidase conjugates for 30 min (dilution 1:1000). Demonstration of peroxidase activity and controls for specificity of the antisera were performed as previously described (Maggi *et al*., 1991). The slides were photographed using a Nikon microphot-FX microscope (Nikon, Kogaku, Tokyo, Japan). For studies in cell cultures, cells were grown on sterile slides and washed twice with phosphate-buffered saline (PBS) pH 7.4 and fixed in 3.7% paraformaldehyde in PBS for 15 min at room temperature, followed by permeabilization in 3.7% paraformaldehyde–PBS containing 0.1% Triton X-100 for 15 min at room temperature. Alternatively, slides were dried overnight and fixed for 10 min in cold acetone. Primary antibodies, appropriately diluted in PBS containing 2% bovine serum albumin (BSA), were added to the slides and incubated overnight at 4%. Slides were washed three times (5 min each) in PBS and incubated at room temperature for 45 min with PBS–2% BSA containing fluoresceinated secondary Ab (dilution 1:100) or IgG peroxidase conjugates (dilution 1:1000). After washing (three times in PBS), slides were examined with a phase contrast microscope equipped with epifluorescence (Nikon microphot-FX microscope). Controls were performed by processing slides lacking the primary Ab or stained with the corresponding non-immune serum.

Endothelin immunoassay

Endothelins were extracted from cell-conditioned media (1.6 ml), by Sep-Pak C_{18} cartridges (Waters-Millipore, Bedford, MA, USA) using methanol/water as mobile phase. The peptide was eluted with methanol/water in the volume ratio 85/15, evaporated to dryness and reconstituted in 250 μl enzyme-linked immunoassay (ELISA) buffer. We used human ET-1 immunoassay QuantiGlo available from R&D Systems. This ELISA shows a cross-reactivity of 27.4% with ET-2 and of 7.8% with ET-3, and a virtually absent cross-reactivity with the bioinactive precursor big-endothelin (<1%). The recovery of ET-1 added to the medium, from the extraction procedure was 90%.

Cell cultures

Human fetal penile cells were prepared from five samples of fetal male external genitalia (11–12 weeks of gestation) obtained after spontaneous or therapeutic abortion. Legal abortions were performed in authorized hospitals, and certificates of approval were obtained from each patient. The use of human fetal tissue for research purposes was approved by the Local Ethical Committee of the University Hospital (Azienda Ospedaliera Careggi, protocol # 6783-04). Human adult penile cells were prepared from cavernosal samples, obtained from three patients undergoing surgical correction for congenital curvature of the penis, as previously described (Filippi *et al*., 2002). Certificates of approval were obtained from each patient and the Local Ethical Committee gave approval for the use of human material. Briefly, penile tissues were mechanically dispersed and treated with 1 mg/ml bacterial collagenase for 15 min at 37°C. Fragments were then collected, washed in PBS and cultured in a mixture 1:1 (vol/vol) of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12 1:1 mix) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/l glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin in a fully humidified atmosphere of 95% air and 5% CO_2. Cells began to emerge within 24–48 h and were used within the fifth passage. LNCaP cells were obtained from American Type Culture Collection (Manassas, VA, USA, USA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% glutamine.

Cell proliferation assay

For growth measurement, $2×10^4$ cells were seeded onto 12-well plates in growth medium. After 24 h, the growth medium was removed, the cells were washed twice in PBS and incubated in phenol red- and serum-free medium

containing 0.1% BSA. After 24 h, cells were maintained in normoxic conditions (95% air, 5% CO_2), and supplemented for 24 h with increasing concentrations of ET-1 (0.01–100 nmol/l) or TGF-β1 (0.03–3 ng/ml) with or without specific ET antagonists or the Ab against TGF-β. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as basal controls. For experiments in hypoxic conditions, cells were grown as described but after 24 h serum starvation, cells were submitted to hypoxia (1.5% O_2, 5% CO_2 and balanced N_2) in the presence or absence of the aforementioned stimuli. After 24 h stimulation, cells were trypsinized and each experimental point was derived from counting on the haemocytometer, and then averaging, at least five different fields for each well. In the same experiment, each experimental point was repeated in duplicate or triplicate. Cell growth results are expressed as percentage (± SEM) of the growth of their relative controls.

Binding assays (endothelin receptors)

Binding assays using [^{125}I]ET-1 (2200 Ci/mmol) in hfPSMC cells were performed as previously described (Maggi et al., 1991). Cells were grown in 24-well dishes. At confluence, cells were washed twice with DMEM, containing 20 mmol/l HEPES, 10 mmol/l $MgSO_4$ and 0.5% BSA, pH 7.4 and were incubated in 200 µl of the same medium at room temperature for 60 min, with fixed concentrations (70 pmol/l) of [^{125}I]ET-1 in the presence or absence of increasing concentrations of the following unlabelled ligands: ET-1 (0.1 nmol/l to 1 µmol/l); ET-3 (0.1 nmol/l to 1 µmol/l); the ETB agonist IRL1620 (0.1 nmol/l to 1 µmol/l); the ETA antagonist BQ123 (0.1 nmol/l to 1 µmol/l). After incubation, cells were extensively washed with ice-cold PBS, 0.1% BSA and solubilized in 0.1 N NaOH, and then the cell-bound radioactivity was determined in a gamma counter. Measurements were performed in triplicate. Cell counts between wells routinely varied by <10%.

Binding assays (androgen receptors)

Cells were grown in Ham's F-12 (without phenol red) supplemented with 10% stripped FBS. At the time of the experiment, cells were harvested and washed with cold TEDMo (Tris–HCl 10 mmol/l pH 7.4, containing EDTA 1.5 mmol/l, dithiothreitol 1 mmol/l and sodium molybdate 10 mmol/l). Cells were then resuspended in TEDGMo (TEDMo + 10% glycerol) and homogenized using an ultraturrax. The homogenate was appropriately diluted and 100 µl, containing 0.88 mg protein, were incubated over night at 4°C in a final volume of 500 µl in TEDGMo with 1 nmol [^{3}H]R1881, in the absence or presence of increasing concentrations (0.1 nmol to 1 µmol/l) of cold R1881. To prevent R1881 binding to progesterone receptor, 1 µmol/l triamcinolone acetonide was added to each tube. Separation of bound and unbound [^{3}H]R1881 was achieved by a 15 min treatment with a 1000 µl suspension of dextran (0.05%)-coated charcoal (0.5%) in Tris–HCl 10 mmol/l pH 7.4, EDTA 1.5 mmol/l at 4°C. The charcoal was pelleted by centrifugation for 10 min at 1500 g and 1300 µl were counted in Instagel plus (Pakard) using a beta counter. Protein content was determined by the method of Bradford using BSA as standard.

5α-reductase activity

The cells were harvested from plates in PBS, pelleted by centrifugation and stored at –80°C. Cell pellets were homogenized in 10 mmol/l potassium phosphate, pH 7.4, 150 mmol/l KCl, 0.1% NOG, 1 mmol/l EDTA, 5 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF) with three short pulses of an Ultraturrax. The concentration of proteins was determined by a published method (Bradford, 1976) using BSA as standard. 5α reductase activity was determined by measuring the conversion of [1,2,6,7-^{3}H$_4$]testosterone to [1,2,6,7-^{3}H$_4$]DHT. The assay was performed by incubating, for 120 min at 37°C, 250 µg of protein in a final volume of 250 µl of assay buffer (10 mmol/l potassium phosphate, pH 7.4, 150 mmol/l KCl, 0.1% NOG) containing increasing concentrations of testosterone (5 nmol–10 µmol/l), a fixed concentration of [1,2,6,7-^{3}H$_4$]testosterone (0.2×10^6 d.p.m.) as tracer and 1 mmol/l NADPH. At the end of the incubation, 750 µl of cold assay buffer were added. Samples were extracted with 3 ml of ethyl acetate. After freezing the aqueous layer, the organic phase was removed and evaporated. Samples were supplemented with T and DHT as carriers (20 µl of a 2 mg/ml solution each) and steroids were separated on thin layer chromatography (TLC) silica plates using dichloromethane/ethyl ether in the volume ratio 85/15 as eluent. The steroids were visible under UV light (enhancer TNS was necessary to identify DHT). Lanes corresponding to T and DHT were scraped

and silica extracted with 2 ml of ethyl acetate. After 30 min, ethyl acetate was counted in a beta counter with 5 ml of scintillation liquid. The percentage of conversion (C) of T into DHT was calculated as follows: C% = [DHT counts/(T counts + DHT counts)]×100.

SDS–PAGE and Western blot analysis

To evaluate the presence of AR, in both hCCSC and hfPSMC, cultured cells grown in RPMI to 70–80% confluence were washed and scraped in PBS. After centrifugation, pellets were extracted in lysis buffer (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.25% NP-40, 1 mmol/l Na$_3$VO$_4$, 1 mmol/l PMSF) on ice for 2 h. After protein measurement, aliquots containing 30 µg of proteins were diluted in reducing 2×SB (Laemmli's sample buffer: 62.5 mmol/l Tris pH 6.8, 10% glycerol, 2% SDS, 2.5% pyronin and 100 mmol/l dithioteithrol) and loaded onto 8% polyacrylamide-bisacrylamide gels. After SDS–PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked for 2 h at room temperature in 5% milk–Tween Tris-buffered saline (TTBS) (0.1% Tween-20, 20 mmol/l Tris, 150 mmol/l NaCl), washed in TTBS and incubated for 2 h with rabbit anti-AR Ab (1:100 in 5% milk–TTBS) followed by peroxidase-conjugated secondary IgG (1:4000 in 5% milk–TTBS). Finally, reacted proteins were revealed by a BM enhanced chemiluminescence system (Roche, Milan, Italy).

Northern blot analysis

Total RNA was extracted from cultured cells with Rneasy Mini Kit Quiagen (Valencia, CA, USA) whereas the Rneasy Midi Kit from the same source was used to extract total RNA from human tissues. RNA concentrations were determined by spectrophotometric analysis at 260 nm. For Northern analysis, 20 µg of total RNA were fractionated in a 1.2% agarose gel containing 8% formaldehyde. RNA was then transferred onto nylon membranes (Hybond-n; Amersham, Milan, Italy) and baked at 80°C for 2 h. Membranes were prehybridized for 1 h and hybridized overnight at 65°C with Church and Gilbert (Church and Gilbert, 1984), buffer solution as described previously (Maggi et al., 1995). The probes for the detection of ET-1, ETA, ETB and ECE-1 mRNA have been previously described (Maggi et al., 1995; Peri et al., 1997). The probe for the detection of TGFβ-1 mRNA was provided by Prof. M.Pinzani (Department of Internal Medicine, Florence University, Florence, Italy). The probes were labelled with deoxycytidine 5'-[α-^{32}P]triphosphate by a random priming kit (Roche, Milan, Italy) and chromatographed (Nu-Clean D25 disposable spun columns; IBI, New Haven, CT, USA) before use. The hybridized nylon membranes were submitted to autoradiography using Hyperfilm-MP (Amersham, Milan, Italy) and Kodak X-Omatic Regular intensifying screens at –80°C for various exposure times.

RT–PCR

500 ng of total RNA were reverse-transcribed and then amplified using the Superscript One Step RT–PCR kit (Invitrogen, Milan, Italy). Oligonucleotide primers were purchased from Invitrogen. The contamination of genomic DNA was excluded by performing 35 cycles of amplification without reverse transcription. The integrity of total RNA was verified by performing RT–PCR for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene.

The sequences of the used primers are as follows: ET-1 sense: 5'-ATGGATTATTTGCTCATGATTTT-3'; ET-1 antisense: 5'-CAGTCTTT-CTCCATAATGTCTTCAGC-3' (Block et al., 1988); ETA sense: 5'-CC-TTTTGATCACAATGACTTT-3'; ETA antisense: 5'-TTTGATGTGGCAT-TGAGCATACAG-3' (Hosoda et al., 1991); ETB sense: 5'-GGACCCATCG-AGATCAAGG-3'; ETB antisense: 5'- AGAATCCTGCTGAGGTGAAGG-3' (Ogawa et al., 1991); AR sense: 5'-ACTCTGGGAGCCCGGAAGCTG-3'; AR antisense: 5'-AATGCTTCACTGGGTGTGGAA-3' (GenBank at NCBI accession no. M000044); phosphodiesterase-5A (PDE5A) sense: 5'-ACC GCTATTCCCTGTTCCTT-3'; PDE5A antisense: 5'-GTAAATGTCCCACC-GTTTCC-3' (Stacey et al., 1998); PDE5A1 sense: 5'-TGGGGTGGAAAAGC-AGTA-3'; PDE5A1 antisense: 5'-AAGAGCAAGATTCGGTGTGG-3' (Lin et al., 2002); PDE5A2 sense: 5'-GCTATGTTGCCCTTTGGAGA-3'; PDE5A2 antisense: 5'-GCAGAGATTTTCCTGGTTGG-3' (Lin et al., 2002); PDE5A3 sense: 5'-GGACACCCAAAGGCAACAT-3'; PDE5A3 antisense: 5'-AAGA-GCAAGATTCGGTGTGG-3' (Lin et al., 2002); 5α-reductase-1 (5α-R1) sense: 5'-GGAATCGTCAGACGAACTCAGTGT-3'; 5α-R1 antisense: 5'-GCATAGCCACACCACTCCATGATT-3' (Berthaut et al., 1997); 5α-R2
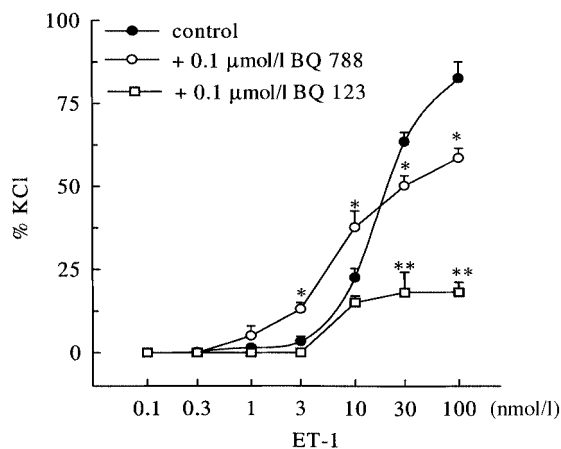
Figure 1. Characterization of endothelin-1 (ET-1) receptors in human corpus cavernosum (CC) strips. Dose–response curve for ET-1 in human CC in the absence (closed circles $n = 6$ in five separate experiments) or presence (open squares, $n = 3$ in two separate experiments) of the specific endothelin-A antagonist, BQ 123 and of the specific endothelin-B antagonist, BQ 788 (open circles, $n = 3$ in two separate experiments). Ordinate: contractile activity, expressed as percentage of the maximal response obtained with KCl (80 mmol/l); abscissa: concentration of the agonist. $*P < 0.05$, $**P < 0.01$ versus control. Data are expressed as the mean $\pm$ SEM. The relative EC_{50} values are reported in the text.

sense: 5′-GAAGCACACGGAGAGCCTGAA-3′; 5α-R2 antisense: 5′-AGCC-CAAGGAAACAAAGTGAG-3′ (GenBank at NCBI accession number M74047); GAPDH sense: 5′-CCATGGAGAAGGCTGGGG-3′; GAPDH antisense: 5′-CAAAGTTGTCATGGATGACC-3′ (Peri *et al.*, 1995).

Statistical analysis

Results are expressed as mean $\pm$ SEM for the indicated number of experiments. Statistical analysis was performed by one-way analysis of variance and paired or unpaired Student's *t*-test when appropriate. $P < 0.05$ was taken as significant. Half-maximal response effective concentration (EC_{50}) and half-maximal response inhibitory concentration (IC_{50}) values were calculated by the computer program ALLFIT (De Lean *et al.*, 1978). Apparent K_m and V_{max} values were calculated by non-linear and linear analysis of data (Michaelis–Menten and Eadie–Scatchard plot) using the Grafit 4.0 software program. The binding data were evaluated quantitatively with non-linear least-squares curve fitting using the LIGAND computer program (Munson and Rodbard, 1980)

Results

Figure 1 shows the effect of ET-1 in isolated preparations of human corpus cavernosum (CC). Increasing concentrations of ET-1 induced a sustained increase in tension with an $EC_{50} = 17 \pm 4$ nmol/l. The ETA antagonist BQ123 almost completely blocked the contractile effect of ET-1, while the ETB antagonist BQ788 induced a biphasic response consisting of a significant enhancement of ET-1 responsiveness at low concentrations of ET-1 (1–10 nmol/l), and in partial antagonism at higher concentrations of ET-1 (30–100 nmol/l). ET-1 was almost 100-fold more potent than the α adrenergic agonist phenylephrine, used as positive control (data not shown; Filippi *et al.*, 2002). Our results clearly indicate, for the first time, that ETA receptor activation is more involved in stimulating CC contractility than ETB. Conversely, the ETB receptors seem to be involved in decreasing the contractile response to ET-1, since the effect of the lower agonist concentrations was enhanced by ETB blockage.

ET-1 and ECE-1 immunoreactivity was observed in human penile cells when we applied the indirect peroxidase method (Figure 2). The specificity of the immunohistochemical staining was demonstrated in CC cells by the absence of labelling in sections incubated with normal rabbit serum (Figure 2F) and by complete absence of staining after pre-adsorption of anti-ET-1 antiserum with synthetic ET-1 (0.1 μmol/l) (Figure 2C). Although specific staining for ET-1 (Figure 2A) and ECE-1 (Figure 2D) was predominant in the endothelial compartment, stromal cells were also positive (Figure 2A and D), in particular in SMC in the arteriolar wall (Figure 2B and E).

Results of RT–PCR experiments showing the expression of specific transcripts for ET-1, its converting enzyme ECE-1 and related receptors in four different human penile tissues and prostate gland, used as positive control (Granchi *et al.*, 2001), are shown in Figure 3A. Figure 3B shows results of Northern analysis on ECE-1 and ET-1 gene expression in three different cultures of human corpus cavernosum stromal cells (hCCSC), prepared as previously described (Filippi *et al.*, 2002). The expression of specific transcripts for ETA and ETB receptors in these cells as well as their biological activity has been previously demonstrated by our group (Filippi *et al.*, 2002). In summary, the results of the present study and of a previous one (Filippi *et al.*, 2002) indicate that human penile tissue expresses genes and proteins for ET-1, ECE-1 and related receptors. Interestingly, we found that ET-1 and ECE-1 expression was not limited to penile EC, but was also present in the muscular compartment.

Figure 4 shows transversal sections of the developing human external genitalia. In these sections, condensed connective tissue forming the corpus cavernosum urethra (CCU) is clearly shown. Between the ventral portion of the remodelling penile urethra and the developing CCU, numerous wide and convoluted blood vessels are present. These blood vessels are invading the CCU to give rise to the forthcoming lacunar spaces. The entire wall of the blood vessels shows an intense positive staining for ET-1 (Figure 4A and B) and ECE-1 (Figure 4C). Hence, as previously observed in adult CC, the expression of ET-1 and its converting enzyme was not limited to the endothelial cells of the developing lacunae, but was also present in the muscular compartment. A negative control section is shown in Figure 4D.

To further characterize the expression of ET-1 in human fetal penile smooth muscle cells (hfPSMC), we cultured these cells from fetal explants of the male external genitalia. Figure 5A shows that virtually all the cells (>90%) were fluorescent when incubated with an Ab against α smooth muscle actin, while they were negative for both cytokeratin and factor VIII (not shown). These findings indicate their smooth muscle origin. Figure 5B shows results from RT–PCR using specific primers for human phosphodiesterase type V (PDE5A) gene and for its three isoforms A1, A2 and A3 (Lin *et al.*, 2002). All three isoforms are present in the human penis (Lin *et al.*, 2002). However, while PDE5A1 and A2 are widely expressed, PDE5A3 is specific for tissues with a cardiac or smooth muscle component including the CC (Lin *et al.*, 2002). Hence, finding PDE5A3 gene expression in hfPSMC further indicates their smooth muscle origin. To fully characterize hfPSMC, we studied the expression of the androgen receptor and 5α-reductase (5α-R) activity, specific markers of DHT-dependent tissues such as the penis, in these cells. We found that hfPSMC not only express transcripts for human AR (Figure 5B), but also specifically bind the synthetic androgen R1881 to the predicted affinity constant for the AR ($K_d = 0.12$ nmol/l, Figure 5C) and express the expected protein, as derived from Western blot analysis (inset of Figure 5C). In addition they express gene (Figure 5B) and protein (Figure 5C) for both 5α-R type 1 (5α-R1) and type 2 (5α-R2) isoforms. In fact, Eadie–Scatchard plot indicated the presence of both isoenzymes with apparent kinetic values for 5α-R1 ($K_m = 6.03$ μmol/l, $V_{max} = 1400$ fmol/mg protein*min) and 5α-R2 ($K_m = 0.2$ μmol/l, $V_{max} = 69$ fmol/mg protein*min) which are compatible with those previously reported in transfected CHO cells (Thigpen *et al.*, 1993). It is interesting to note that 5α-R2 expression
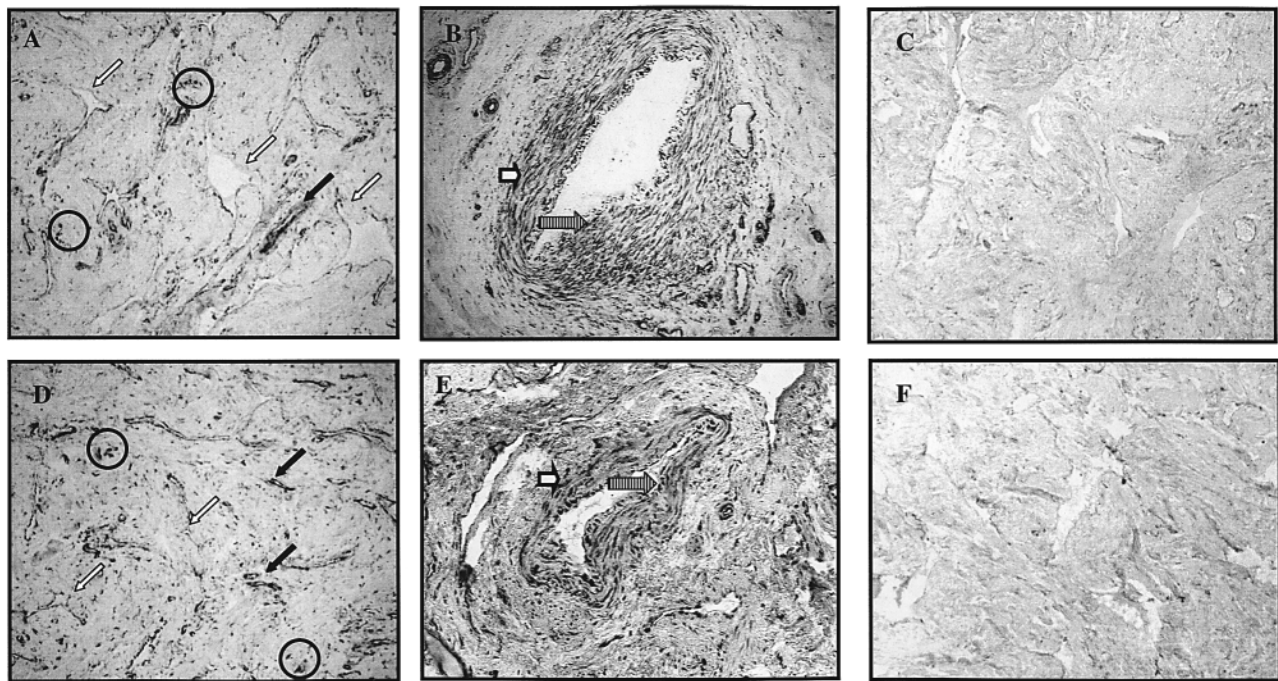
Figure 2. Transversal sections of human adult corpus cavernosum (CC). Black arrows indicate the lacunar spaces. White arrows indicate the blood vessels. Hatched arrows indicate the endothelial cells (EC); white arrowheads indicate the smooth muscle cells (SMC) in an arteriolar wall. (**A** and **D**) Immunostaining for endothelin-1 (ET-1) (**A**) and ET-1-converting enzyme (ECE-1) (**D**). Blood vessels and lacunar spaces are intensely positive. Black circles mark clusters of dispersed stromal cells immunopositive for ET-1 (**A**) and its converting enzyme (**D**). (**B** and **E**) Arteriolar wall. Both EC (hatched arrows) and SMC (white arrowheads) are stained with antibodies against ET-1 (**B**) and ECE-1 (**E**). (**C** and **D**) Control sections obtained after pre-absorption with ET-1 (100 nmol/l) (**C**) or omitting the primary Ab for ECE-1 (**F**). Original magnifications: **A**, **C**, **D**, ×120; **B**, **E**, **F**, ×200.
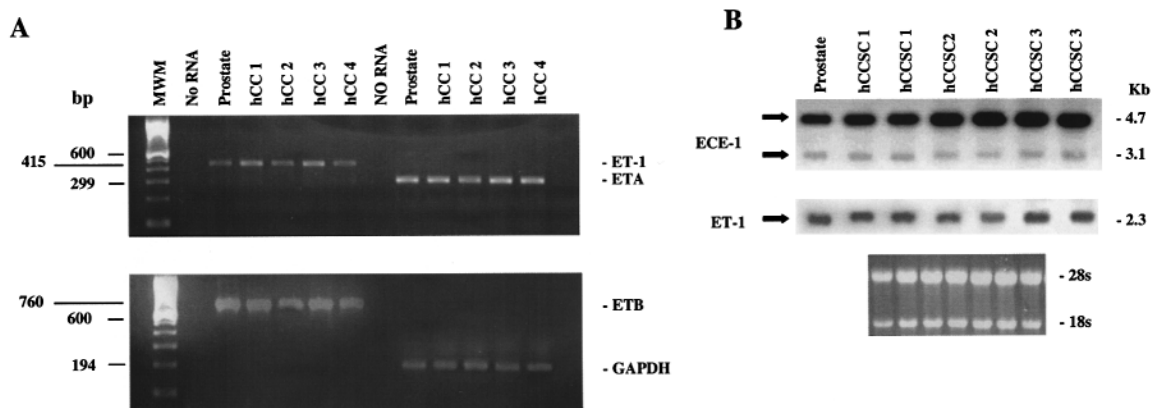
Figure 3. Expression of endothelin-1 (ET-1), ET-1 converting enzyme (ECE-1) and related receptors in human corpus cavernosum (hCC) from different patients and in different preparations of human CC stromal cells (hCCSC). (**A**) RT–PCR. The products are derived from total RNA using specific primers for ET-1 and endothelin-A (upper panel), and for endothelin-B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (lower panel) in different corpus cavernosum specimens (hCC1-4). Human prostate total RNA was used as positive control. GAPDH mRNA amplification was performed to verify the integrity of the extracted total RNA. Molecular weight markers (MWM) are indicated on the left. (**B**) Gene expression of ECE-1 and ET-1 in hCCSC, as detected by Northern analysis. Total RNA extracted from human prostate was used as positive control. Each lane was loaded with 20 µg of total RNA. Corresponding ethidium bromide staining of the gel is shown below the blot. Total RNA from three different preparations of hCCSC cells was employed (hCCSC1–3).

is specific for the DHT-dependent tissues, such as the developing human male external genitalia (S.Kim *et al.*, 2002). Hence our data, taken together, clearly demonstrate that hfPSMC are of smooth muscle origin and that they are derived from an androgen-dependent tissue such as the developing human penis.

As expected from immunohistochemical studies, hfPSMC show positive staining for ET-1 in the cytoplasm (Figure 6A) and release ET-1 in the spent medium as a function of time (Figure 6B). They also express the ET-1 gene as observed by Northern analysis (Figure 7A, C and D). ET-1 gene expression was transiently stimulated by TGF-β1 exposure (1 ng/ml, Figure 7A). Maximal (2-fold)

stimulation was obtained after 1 h incubation (Figure 7A). Accordingly, incubation with TGF-β1 (1 ng/ml) stimulated a sustained increase in ET-1 protein in the conditioned medium (Figure 7B). A sustained increased in ET-1 gene expression was also noticed in hfPSMC after short-term exposure to ET-1 itself (100 nmol/l, Figure 7C) and IL-1α (50 ng/ml; Figure 7D). To verify whether hfPSMC express not only ET-1 but also its related receptors, we performed RT–PCR studies in different cell preparations. Specific transcripts for ETA and, to a lesser extent, for ETB receptors were detected in four different preparations of hfPSMC (Figure 8A). In agreement with the data obtained from RT–PCR experiments, hfPSMC
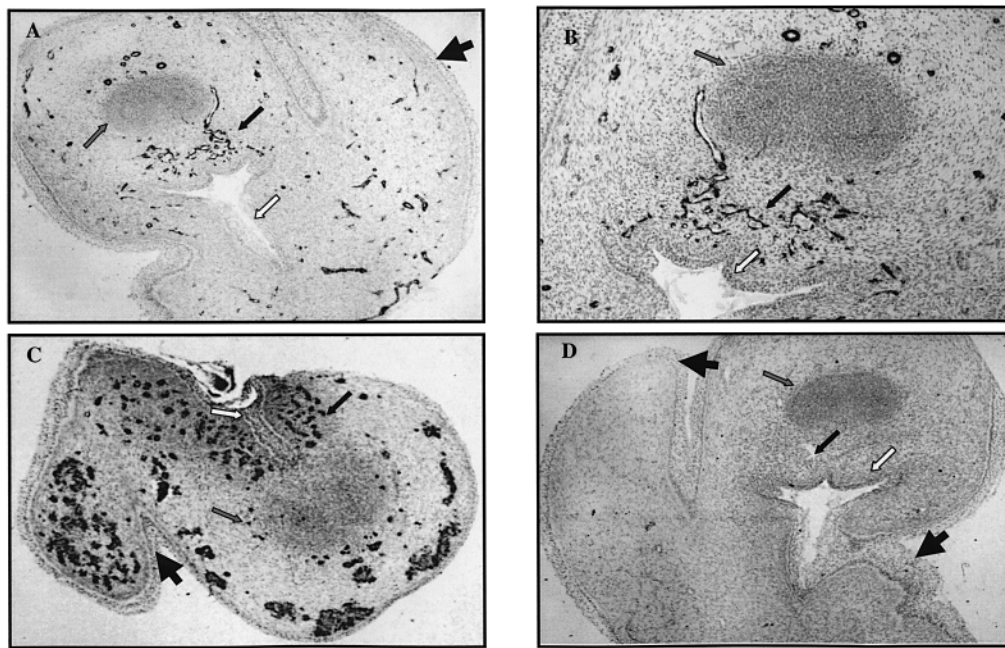
Figure 4. Transverse sections of the human male external genitalia at the 11th week of gestation. Gray arrows indicate the condensed connective tissue forming the corpus cavernosum (CC) urethrae; black arrows indicate convoluted blood vessels invading the CC urethrae; white arrows indicate the penile urethra. Black arrowheads indicate the developing scrotal swellings. (**A**) Immunostaining for endothelin-1 (ET-1): positive cells are present in the vessel wall and scattered throughout the mesenchyme (×80). (**B**) Enlarged area from **A** (×150). (**C**) Immunostaining for ET-1-converting enzyme (ECE-1): positive cells are distributed as in **A** (×80). (**D**) Negative control (immunoabsorption of the anti-ET-1 antiserum with the synthetic peptide, 100 nmol/l) (×80).

specifically bound labelled ET-1 (Figure 8B) with high capacity (B_{max} = 46 600 ± 3900 sites/cell) and high affinity (K_d = 0.93 ± 0.09 nmol/l). A similar affinity was observed for the specific ETA antagonist BQ123 (K_d = 0.67 ± 0.06 nmol/l). Conversely, the ETB agonists, ET-3 and IRL1620, displaced labelled ET-1 with lower potency (K_d = 263 ± 72 mol/l and 1804 ± 540 nmol/l respectively). These findings indicate that hfPSMC predominantly express the ETA receptors. To study the biological activity of ET receptors in hfPSMC, we conducted growth studies. As shown in Figure 9, a 24 h exposure to increasing concentrations of ET-1 significantly decreased hfPSMC proliferation, with an IC_{50} = 0.14 ± 0.016 nmol/l. Incubation of hfPSMC cells with the ETA antagonist BQ123 (100 nmol/l), but not with the ETB antagonist BQ788 (100 nmol/l), completely blocked the anti-proliferative effect of ET-1 (100 nmol/l). Because we noticed that a short-term exposure of hfPSMC to ET-1 (100 nmol/l) stimulated TGF-β1 gene expression (Figure 10A), and because TGF-β1 is a well-known inhibitor of CC cell proliferation, we tested the hypothesis that TGF-β1 mediates the anti-proliferative effect of ET-1. As shown in Figure 10B, increasing concentrations of TGF-β1 strongly decreased hfPSMC growth. The effect of 0.3 ng/ml of TGF-β1 was completely blocked by an Ab against TGF-β but not by the ETA antagonist BQ123. Conversely, the anti-proliferative effect of ET-1 (100 nmol/l) was completely prevented by the Ab against TGF-β, while the Ab alone was without effect (not shown).

Because penile tissue is exposed to low oxygen tension most of the time, we investigated the effect of hypoxia on the expression of ET-1 and its cognate receptors. We therefore incubated hfPSMC at an oxygen tension of 1.5% O_2, mimicking the physiological oxygenation during penile flaccidity, for various times. Results are reported in Figure 11. ET-1 mRNA expression decreased progressively during serum starvation both in normoxic and hypoxic conditions, as previously observed (Granchi *et al.*, 2001). Interestingly, we found that, at the latest time point, hypoxia stimulated an increase in ET-1 gene expression (Figure 11A). Also ET-1 protein increased after 24 h hypoxia, but such an increase reached statistical significance only

after 48 h (Figure 11B). Hypoxia not only stimulated ET-1 expression but also induced a sustained 6-fold increase in ETB mRNA, while the expression of the ETA receptors was slightly, but not significantly, decreased (Figure 11C). Because hypoxia changed the expression pattern of ET-1 receptors, we repeated growth curves by incubating hfPSMC with ET-1 at low oxygen tension. We found that in this experimental condition, ET-1 stimulated hfPSMC proliferation with an IC_{50} = 7.6 ± 5 pmol/l (Figure 12). The stimulatory effect of ET-1 was completely abrogated by the ETB antagonist BQ788 (100 nmol/l), and only partially decreased by the ETA antagonist BQ123 (100 nmol/l).

Discussion

ET-1 is the most potent stimulator of penile smooth muscle cell contractility, as demonstrated by the present and previous studies (Saenz De Tejada *et al.*, 1991; Holmquist *et al.*, 1992; Filippi *et al.*, 2002). In this study, we originally demonstrated that ET-1 induces human CC contractility, acting through the ETA subtype of ET-1 receptors, while the ETB subtype apparently has the opposite effect. However, the main finding of this study is that human penile smooth muscle cells are not only a paracrine target for ET-1, but also represent an autocrine source. In fact, combined immunohistochemical and in-vitro culture studies indicate that adult penile smooth muscle cells, as well as endothelial cells, express ET-1 and its specific converting enzyme ECE-1. Our results are in partial agreement with those of Saenz De Tejada *et al.* (1991) who showed ET-1 immunopositivity in the trabecular smooth muscle cells of the human penis. However, the same authors were unable to demonstrate ET-1 gene expression in cultured stromal cells by Northern analysis (Saenz De Tejada *et al.*, 1991).

The aforementioned pattern of ET-1 expression not only characterizes the adult CC but is also present in the developing tissue. We report that cultures of hfPSMC express ET-1 and ECE-1 genes, and that ET-1 protein is released over time in the conditioned medium in
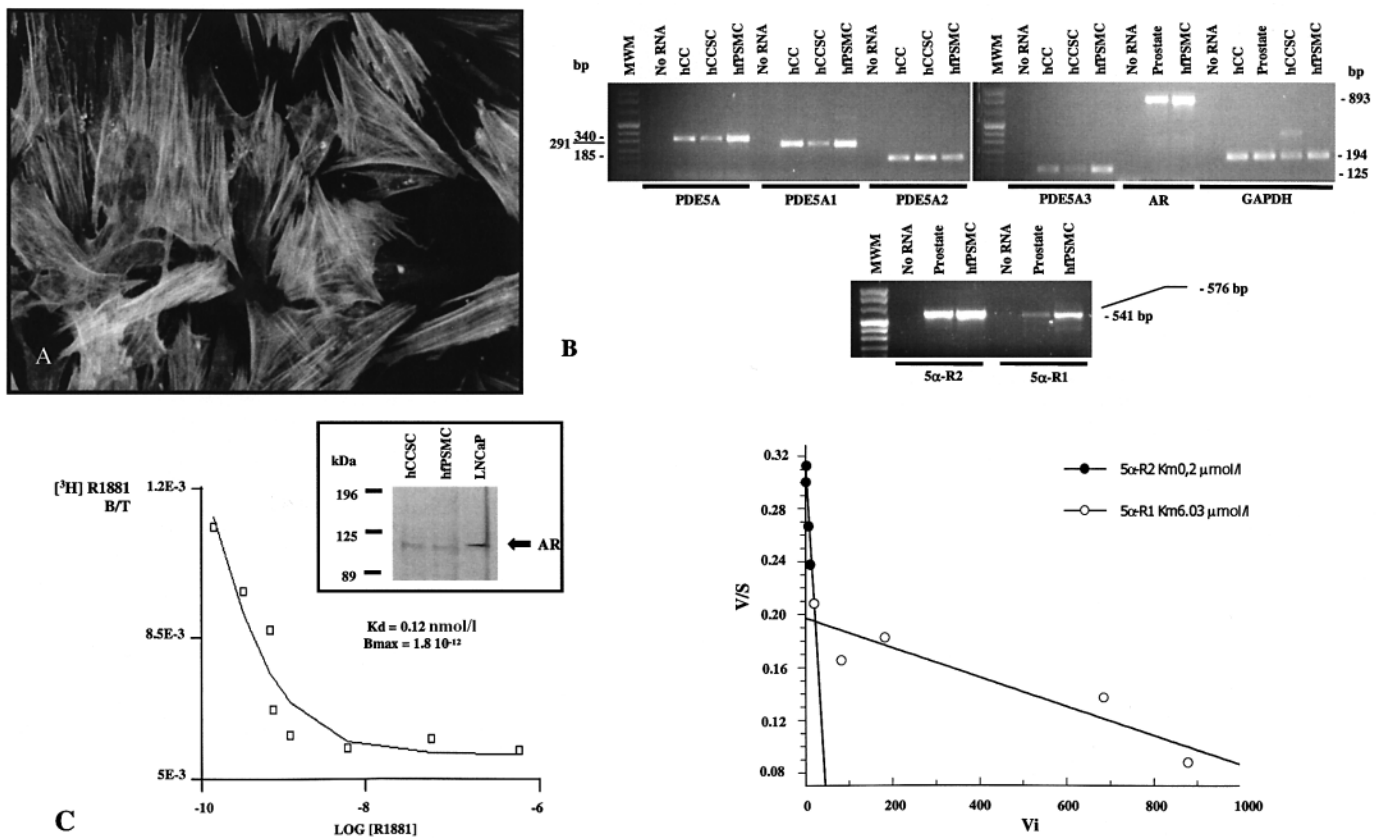
Figure 5. Characterization of human fetal penile smooth muscle cells (hfPSMC) cells. (**A**) α-smooth muscle actin immunofluorescence. Note the typical morphological features of SMC and the presence of the stress fibres. (**B**) Phosphodiesterase type 5A (PDE5A) and its major isoforms (A1, A2, A3), androgen receptor (AR) and 5α reductase (5α-R) type 1 (5α-R1) and 2 (5α-R2) gene expression in hfPSMC as detected by RT–PCR. The products are derived from total RNA using PDE5A, PDE5A1, PDE5A2, PDE5A3, AR, 5α-R1 and 5α-R2 specific primers. Human prostate, corpus cavernosum and hCCSC total RNA were used as positive controls. GAPDH mRNA amplification was performed to verify the integrity of the extracted total RNA. The molecular weight markers (MWM) are indicated on both sides of the gels. (**C**) Expression of AR in hfPSMC. Homologous competition curve for [3H]R1881: ordinate: B/T, bound to total ratio for [3H]R1881; abscissa: total concentration (molar) of labelled and unlabelled R1881. Inset: Western blot detection of AR protein in hCCSC hfPSMC and LNCaP cells used as positive control. All lysates were obtained as described in Materials and methods, and probed for AR expression with anti-androgen receptor polyclonal Ab (1:100). A single band of ~110 kDa (arrow) was present in all cell preparations. MWM (kDa) is indicated to the left of the blot. (**D**) Eadie–Scatchard plot of estimated initial velocities over substrate concentration (V/S) against velocity (Vi) for 5α reductase hfPSMC homogenate. The plot is clearly non-linear, indicating the presence of 5α-R1 (open circles) and 5α-R2 (closed circles) isoenzymes.
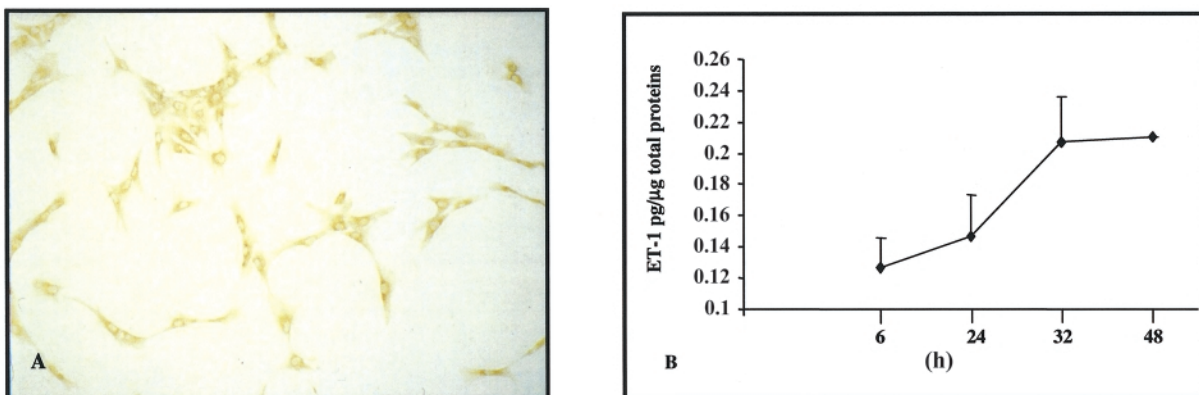

Figure 6. Expression of endothelin-1 (ET-1) protein by human fetal penile smooth muscle cells (hfPSMC). (**A**) Immunolocalization of ET-1 in hfPSMC. Positivity is present in the majority of cells and limited to the cytoplasm. (**B**) Time course of ET-1 release (pg/μg total protein) in conditioned medium without serum. ET-1 was measured using an enzyme-linked immunosorbent assay at the indicated time points. ET-1 release increases over time. Data are mean ± SEM of triplicate determinations.

similar amounts to those previously reported for other smooth muscle (Markewitz *et al*, 2001) or epithelial cells (Markewitz *et al*., 1995). Besides expressing ET-1, hfPSMC display several important features. They express all the genes for the presently characterized isoforms

of PDE5A, the human enzyme involved in cGMP breakdown and in sildenafil action at the CC level, including PDE5A3. This isoform is supposed to be specific for cardiac and smooth muscle cells (Lin *et al*., 2002). Moreover, hfPSMC express genes and proteins for the
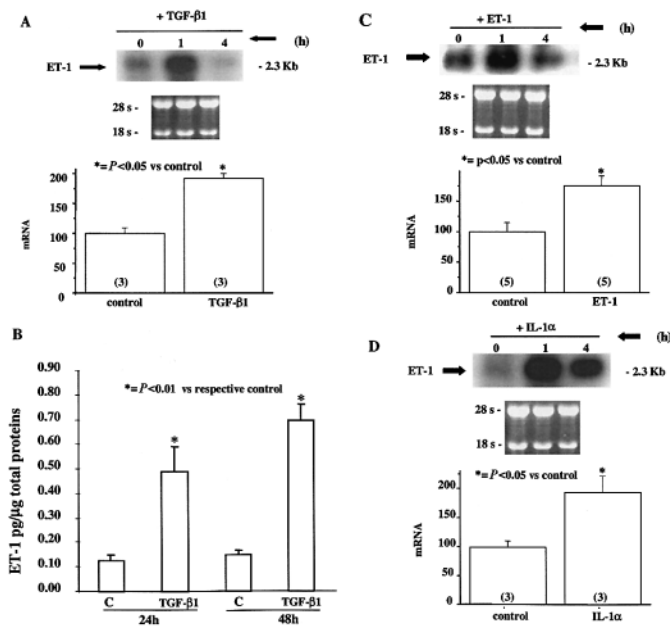
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Figure 7. Effect of the different stimuli on endothelin-1 (ET-1) expression in human fetal penile smooth muscle cells (hfPSMC). (**A**) Effect of incubation, for different times (1 and 4 h) in serum-free medium, with transforming growth factor-β1 (TGF-β1) (1 ng/ml) on ET-1 mRNA expression. Each lane was loaded with 20 μg of total RNA. Below the blot are shown the corresponding ethidium bromide staining of the gel and the densitometric analysis of ET-1 gene expression (1 h stimulation) in the indicated numbers of experiments. Data are expressed as percentages of their relative control. (**B**) Effect of TGF-β1 (1 ng/ml) on ET-1 secretion at different time points (24 and 48 h). ET-1 was measured in conditioned medium without serum by a specific ELISA and expressed as pg/μg total protein. TGFβ-1 significantly increased ET-1 release at all the time points ($P < 0.001$). Data are mean ± SEM of triplicate determinations. (**C**) Effect of incubation for different times (1 and 4 h) in serum-free medium with ET-1 (100 nmol/l) on ET-1 mRNA expression. Results are expressed as in **A**. (**D**) Effect of incubation for different times (1 and 4 h) in serum-free medium with IL-1α (50 ng/ml) on ET-1 mRNA expression. Results are expressed as in **A**.

androgen receptor and for 5α-reductase type 2, selective markers of the developing male external genitalia (S.Kim *et al.*, 2002). In addition, they express genes and proteins for ET-1 receptors. Therefore, hfPSMC are a useful tool for studying the interplay between androgens and the ET-1 system and their mutual interaction with PDE5A isoforms. In addition, hfPSMC could represent an alternative source of penile smooth muscle cells for studying the erectile process in humans. The availability of human adult penile erectile tissue is restricted and in the vast majority of cases, the tissue available for research purposes is derived from patients having important diseases such as diabetes, arteriosclerosis, induratio penis plastica or from individuals undergoing gender reassignment operations after estrogen treatment. Such pathological conditions might *per se* impair the physiological function of penile smooth muscle cells. Furthermore it is not easy to obtain pure cultures of adult penile smooth muscle cells, i.e. without fibroblast contamination. Conversely, our preparations of hfPSMC are virtually all positive for α smooth muscle actin and show the biochemical features of penile contractile cells.

We observed that ET-1 gene and protein expression in hfPSMC could be triggered by several factors, such as TGF-β1 and hypoxia. In addition, inflammatory factors such as IL-1α, and the same ET-1 itself, also induced a transient increase in ET-1 transcripts. Such regulation of ET-1 expression is in perfect agreement with previous observations of ET-1 regulation in another area of the male genital tract, such as the prostate gland (Granchi *et al.*, 2001), and in other smooth muscle cells (Sugo *et al.*, 2001). TGF-β1 is a growth factor very much involved in penile tissue remodelling (Bivalacqua *et al.*, 2001) and fibrosis (El-Sakka *et al.*, 1997; Moreland *et al.*, 1998; Nehra *et al.*, 1999), and is known to decrease penile growth (Gelman *et al.*, 1998). In this study, we confirm that TGF-β1 dose-dependently inhibits penile smooth muscle cell proliferation. ET-1 also impairs hfPSMC growth. The ETA subtype of ET-1 receptors apparently mediates this effect, because BQ123 (an ETA antagonist), and not BQ788 (an ETB antagonist), completely reverses the anti-proliferative activity of ET-1. Because an Ab against TGF-β counteracts the inhibitory effect not only of TGF-β1 but also that of ET-1, we speculate that the anti-proliferative effect of ET-1 is at least partially
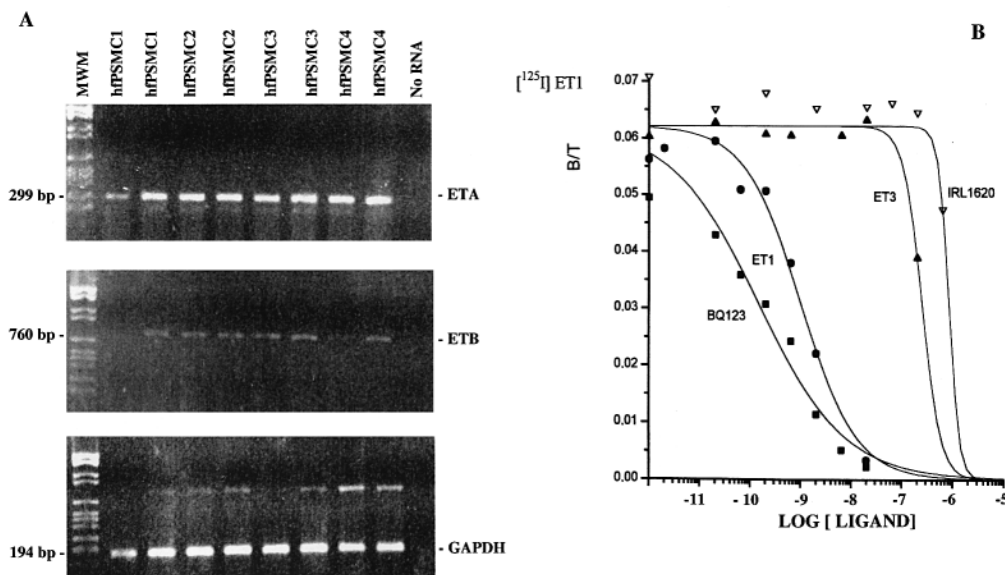

Figure 8. Endothelin-A (ETA) and endothelin-B (ETB) expression in human fetal penile smooth muscle cells (hfPSMC). (**A**) RT–PCR products from total RNA of different preparations of hfPSMC (1–4) using ETA (upper), ETB (middle) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (lower) specific primers. GAPDH mRNA amplification was performed to verify the integrity of the extracted total RNA. Molecular weight markers (MWM) are indicated on the left side of the blots. (**B**) Competition curves between [^{125}I]ET-1 and unlabelled ET-1 (closed circles), ET-3 (closed triangles), IRL1620 (open triangles) and BQ123 (closed boxes) in hfPSMC. Ordinate: B/T, bound to total ratio for [^{125}I]ET-1; abscissa: total concentration (molar) of ligands.
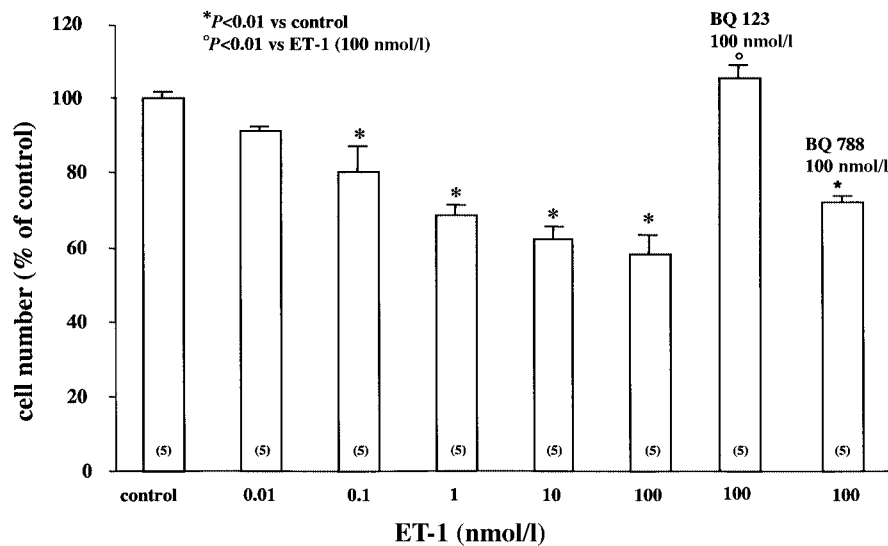
Figure 9. Effect of endothelin-1 (ET-1) and specific receptor antagonists on human fetal penile smooth muscle cell (hfPSMC) proliferation. After 24 h of serum starvation, hfPSMC were treated for an additional 24 h with increasing concentrations (0.01–100 nmol/l) of ET-1. Experiments were also performed with the maximal concentration of ET-1 (100 nmol/l) with or without the endothelin-A (ETA) (BQ123, 100 nmol/l) and endothelin-B (ETB) (BQ788, 100 nmol/l) receptor antagonists. The ET-1 dose-dependently decreased hfPSMC growth. ET-1-induced anti-proliferative activity was reversed by the simultaneous administration of BQ123, but not by BQ788. Results were derived from the analysis of five independent experiments in two separate preparations of hfPSMC.
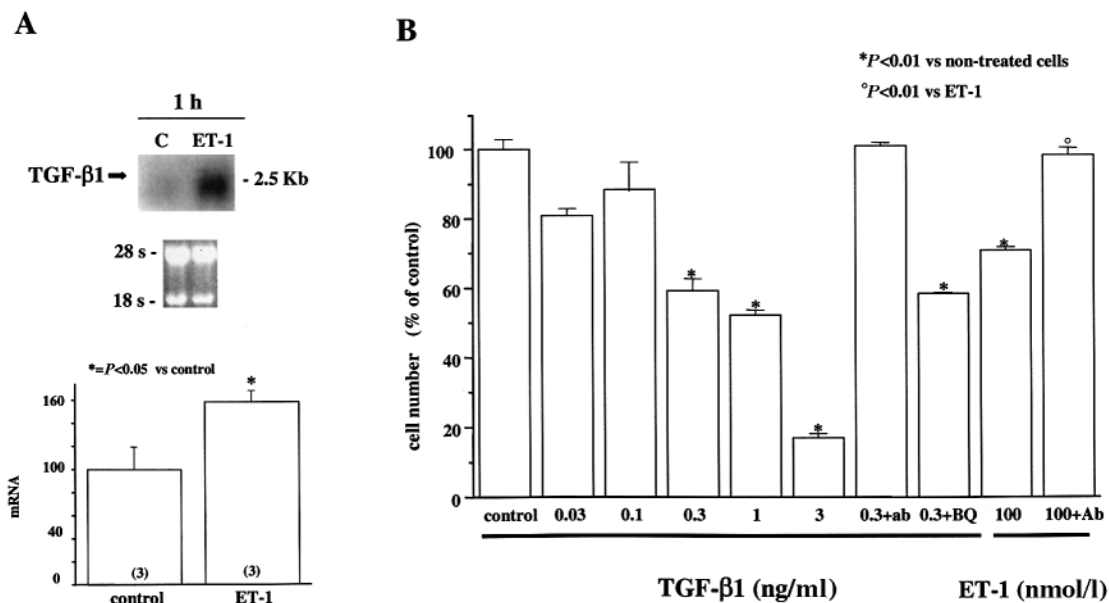

Figure 10. Effect of transforming growth factor-β1 (TGF-β1) on human fetal penile smooth muscle cells (hfPSMC). (**A**) Effect of endothelin-1 (ET-1) on TGF-β1 gene expression. Experiments were performed by stimulating hfPSMC with ET-1 (100 nmol/l) for 1 h in serum-free medium. Each lane was loaded with 20 µg of total RNA. The blot was hybridized with TGFβ-1 cDNA probe labelled with ^{32}P. Below the blot is shown the corresponding ethidium bromide staining of the gels and the densitometric analysis of TGF-β1 gene expression. Data are expressed as percentages of control. The number of experiments is indicated in parentheses. (**B**) Effect of TGF-β1 on hfPSMC proliferation. After 24 h serum starvation, hfPSMC were treated for an additional 24 h with increasing concentrations (0.03–3 ng/ml) of TGF-β1 or with TGF-β1 (0.3 ng/ml) and the Ab against TGFβ or the endothelin-A (ETA) antagonist BQ123 (100 nmol/l). While the anti-TGFβ Ab completely reversed the anti-proliferative effect of TGF-β1, the ETA antagonist was without effect. In the right portion of the histogram the effect of ET-1 (100 nmol/l), with or without the Ab against TGFβ, is shown. The anti-TGFβ Ab also completely reversed the anti-proliferative effect of ET-1.

mediated by TGF-β. Accordingly, ET-1 increased TGF-β1 gene expression in hfPSMC.

Another important observation of the present study is the effect of hypoxia on ET-1 and related receptor expression. The human penis and its CC remain flaccid for at least 21 out of 24 h daily. During this time, the flaccid penis is exposed to venous-like oxygen tension and, as a consequence, to a situation of relative hypoxia (CC oxygen tension is ~25–40 mmHg) (Brindley *et al.*, 1983; Kim *et al.*, 1993;

Sattar *et al.*, 1995; Brown *et al.*, 1997). Only during erections does the increased arterial blood flow allow CC oxygen tension to achieve values of ~90–100 mmHg (Kim *et al.*, 1993; Sattar *et al.*, 1995; Nehra *et al.*, 1996; Brown *et al.*, 1997). We found that prolonged (24 h) exposure of penile smooth muscle cells to an oxygen tension similar to that of the flaccid state significantly increased ET-1 gene expression and, later on, protein release. The same results have been obtained in endothelial cells (Kourembanas *et al.*, 1991) and
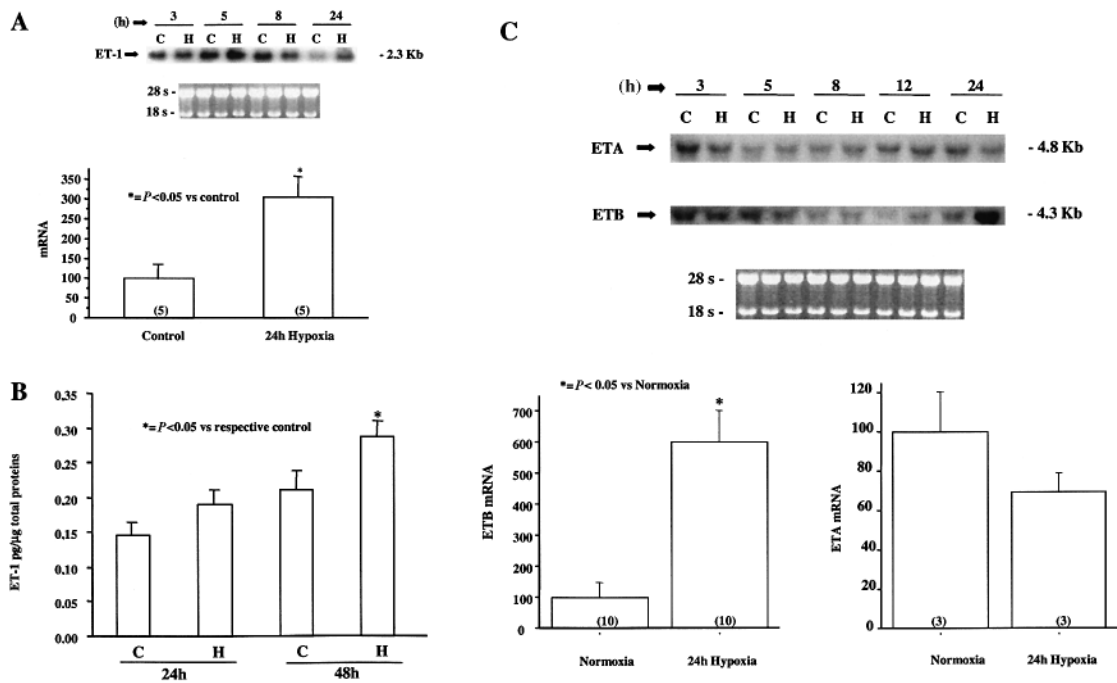
Figure 11. Effect of hypoxia (1.5% O_2, 5% CO_2 and balanced N_2) on endothelin-1 (ET-1) and related receptor expression in human fetal penile smooth muscle cells (hfPSMC). C = control, normoxia; H = hypoxia. (**A**) Northern analysis of ET-1 mRNA expression at the indicated time points of hypoxia. Every lane was loaded with 20 μg of total RNA. Below the blot are shown the corresponding ethidium bromide staining of the gel and the densitometric analysis of ET-1 gene expression (24 h hypoxia) in the indicated numbers of experiments. Data are expressed as percentages of control. The number of experiments is indicated in parentheses. (**B**) Effect of hypoxia (24 and 48 h) on ET-1 release (pg/μg total protein) in conditioned medium without serum from hfPSMC. ET-1 was measured using an enzyme-linked immunosorbent assay kit. Data represent mean ± SEM of triplicate determinations. (**C**) Northern analysis of endothelin-A (ETA) (upper blot) and endothelin-B (ETB) (lower blot) mRNA expression at the indicated time points of hypoxia. Every lane was loaded with 20 μg of total RNA. Below the blots, the corresponding ethidium bromide staining and the densitometric analysis for the ETB (left histogram) and the ETA (right histogram) gene expression (24 h hypoxia) are shown in the indicated numbers of experiments. Data are expressed as percentages of their relative controls.
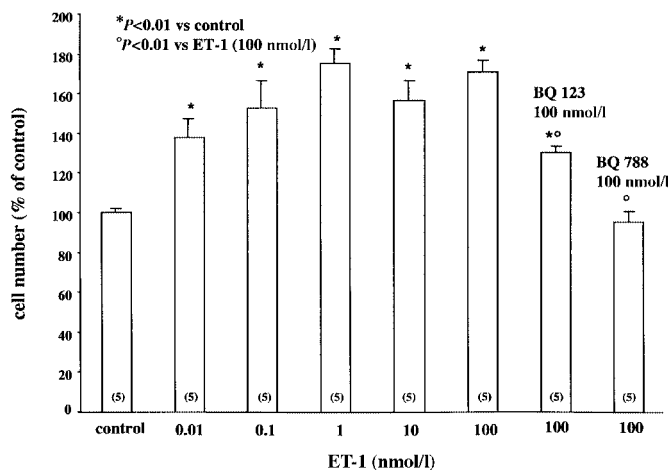

Figure 12. Effect of endothelin-1 (ET-1) and specific receptor antagonists on human fetal penile smooth muscle cell (hfPSMC) proliferation after 24 h of an experimental hypoxic condition. After 24 h of serum starvation, hfPSMC were incubated in hypoxic conditions (1.5% O_2, 5% CO_2 and balanced N_2) and treated for 24 h with increasing concentrations (0.01–100 nmol/l) of ET-1 or with the maximal concentration of ET-1 (100 nmol/l) and the endothelin-A (ETA) (BQ123, 100 nmol/l) or the endothelin-B (ETB) (BQ788, 100 nmol/l) receptor antagonists. ET-1 dose-dependently increased hfPSMC growth. ET-1-induced proliferation was completely reversed by the simultaneous administration of BQ788, but only attenuated by BQ123. Results were derived from the analysis of five independent experiments in three separate preparations of hfPSMC.

cardiomyocytes, where it was related to the presence of a hypoxia-inducible factor-1 (HIF-1) binding site in the ET-1 promoter (Kakinuma *et al.*, 2001). We also found that lowering oxygen tension not only increases ET-1 production but also affects the responsiveness of hfPSMC to ET-1 itself. During normoxia, hfPSMC responded to ET-1 with decreased proliferation, whereas in experimental hypoxic conditions, ET-1 stimulated cell growth. This effect might be due to a changed pattern of ET-1 receptor expression, related to the protracted hypoxic state. We observed that 24 h of hypoxia increased ETB mRNA expression without significantly altering ETA mRNA abundance, a result consistent with previous observations in other cell types such as astrocytes (Shibaguchi *et al.*, 2000). Chronic hypoxia in rat lungs induces an increased responsiveness of ETB receptors in terms of vasodilatation, through NO release, and bronchoconstriction (Muramatsu *et al.*, 1999; Lal *et al.*, 2000), most probably related to increased ETB mRNA expression (Soma *et al.*, 1999). Accordingly, in a genetic rat model of ETB deficiency, hypoxia has been shown to induce an increased vasoconstrictor response to ET-1 in lung vessels, apparently due to decreased endothelial NO synthase (eNOS) activity and NO production (Ivy *et al.*, 2001). Also in our model, the changing proliferative response to ET-1 was related to a hypoxia-induced ETB up-regulation, because it was completely abolished by an ETB antagonist (BQ788) and only partially affected by an ETA antagonist (BQ123). Our finding of an ETA-mediated inhibition and an ETB-mediated stimulation of cell growth is in apparent contrast with observations made in other adult myoid cells such as rat testicular peritubular (Santiemma *et al.*, 1996) and hepatic stellate cells (Pinzani *et al.*, 1996), but is in agreement with a recent observation from our group in human fetal GnRH neurons (Maggi *et al.*, 2000b). It is

possible that the receptor-specific effect of ET-1 on proliferation is dependent upon several factors, including cell specificity, pattern of signal transmission or degree of differentiation.

In conclusion, our study demonstrates that smooth muscle cells of the human penis express ET-1 and its receptors not only during adulthood, but also during fetal life. Especially during the development of male external genitalia, ET-1 and related receptors may have a role in modulating blood flow supply and tissue remodelling, regulating smooth muscle cell contraction and proliferation. It is interesting to note that penile erection has been measured by ultrasound as early as at weeks 11–12 of gestation (Pedreira *et al.*, 2001) and that discrete episodes of nocturnal penile erections are clearly evident also at term gestation (Shirozu *et al.*, 1995). These fetal penile erections may have the same role as nocturnal erection during adulthood, i.e. increasing the penile blood flow and therefore cavernous oxygen tension. If these results are confirmed also in the adult penis, they may explain the detrimental effects of several cardiovascular or neurological diseases that impair penile erection (even during sleep) by substantially decreasing penile oxygenation for prolonged time (i.e. >24 h). In fact, the penis is a rather exceptional vascular bed, with its oxygenation dependent upon erectile activity. In normal men, discrete episodes of penile erection are present at least every 24 h, during eye rapid movement sleep. These episodes are three to five per night and last 20–40 min, allowing a sufficient amount of oxygen to be delivered to the CC cells. The pathological absence of such episodes determines a prolonged flaccidity and hypoxia. The present study may indicate that a decreased penile oxygenation for a prolonged time will in turn induce ET-1 expression and SMC proliferation, possibly perpetuating the vascular damage.

Acknowledgements

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